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Piperlongumine selectively suppresses ABC-DLBCL through inhibition of NF-κB p65 subunit nuclear import

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ABSTRACT

Constitutive NF-κB activation is required for survival of activated B cell-like subtype of diffuse large B cell lymphoma (ABC-DLBCL). However, current NF-κB targeting strategies lack cancer cell specificity. Here, we identified a novel inhibitor, piperlongumine, features direct binding to NF-κB p65 subunit and suppression of p65 nuclear import. This was accompanied by NF-κB reporter activity suppression and NF-κB target gene downregulation. Moreover, mutation of Cys^{38} to Ser in p65 abolished this effect of piperlongumine on inhibition of p65 nuclear import. Furthermore, we show that piperlongumine selectively inhibited proliferation and induced apoptosis of ABC-DLBCL cells. Most notably, it has been reported that piperlongumine did not affect normal cells even at high doses and was nontoxic to animals. Hence, our current study provides new insight into piperlongumine's mechanism of action and novel approach to ABC-DLBCL target therapy.

1. Introduction

Diffuse large B cell lymphoma (DLBCL) is considered to be the most common subtype of non-Hodgkin Lymphoma, accounting for 30%–40% of all newly diagnosed cases [1]. The most widely used molecular subclassification divides DLBCL into three subtypes according to gene expression profiling, including germinal center B cell-like (GCB) DLBCL, activated B cell-like (ABC) DLBCL, and primary mediastinal B cell lymphoma (PMBL) [2]. The molecular DLBCL subtypes not only differ with respect to their gene expression patterns but also have significantly different overall survival rates. The GCB subtype patients have a significantly better overall survival compared with the ABC subtype patients. The 5-year survival rate of ABC-DLBCL patients only is 35%, which reflecting the aggressive clinical behavior of ABC-DLBCL cells [3]. Development of novel targeted therapies are urgently needed for ABC-DLBCLs, which are the most chemoresistant DLBCLs.

The hallmark of ABC-DLBCL subtype cells is the constitutive anti-apoptotic NF-κB activity but not GCB-DLBCL. Gene expression profiling of cancer biopsy samples and cell lines revealed preferential expression of NF-κB target genes in ABC-DLBCL compared with GCB-DLBCL [4]. NF-κB activation is induced by chronic active B-cell receptor (BCR) signaling or constitutive MYD88 activity [5]. Oncogenic mutations that contribute to NF-κB activation have been identified in critical components of both pathways [6]. Under normal conditions, NF-κB stays in an inactive state in the cytoplasm as a heterotrimer consisting of p50, p65 and IκB-α [7]. However, the upstream oncogenic mutations cause the degradation of IκB-α in ABC-DLBCL. The p65 subunit is then release from the cytoplasm and translocate to the nucleus, where it binds to a specific consensus sequence in the DNA and activates the transcript of more than 500 genes [8]. The targeted therapies for ABC-DLBCL have largely focused on the inhibition of upstream protein kinase. However, most oncogenic mutations in ABC-DLBCL occur further downstream revealing that the upstream protein kinases may not be optimal targets [9]. Hence, specific small molecule inhibitors

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suppress the NF-κB p65 subunit nuclear import could be beneficial for the treatment of ABC-DLBCL.

Piperlongumine, a natural isolated from Long pepper, has been used widely in Indian and Chinese traditional medicine [10]. It has captured a great deal of attention because of its potent anti-tumor activity. Notably, recently report demonstrated that piperlongumine could selectively kill multiple types of cancer cells but not noncancerous cell types even at high doses [11]. Furthermore, piperlongumine has favorable toxicity profile and does not appear to significantly affect any biochemical, hematologic and histopathologic parameters in animal models. More recently, it has been shown that piperlongumine could suppress constitutive NF-κB activity in prostate cancer cells [12]. Unfortunately, its detailed molecular mechanism of NF-κB inhibition is still in much debate and remains to be elucidated. Thus, the goal of this study was to determine whether piperlongumine can more selectively suppress ABC-DLBCL cells, which have constitutive activation of NF-κB pathway. If so, we investigate in detail the effect of piperlongumine on NF-κB pathway regulation.

In this report, for the first time, we demonstrated that piperlongumine could selectively inhibit the proliferation of ABC-DLBCL via directly blocking NF-κB p65 subunit nuclear import. We found that piperlongumine inhibited NF-κB activation and NF-κB regulated gene products, leading to apoptosis of ABC-DLBCL cells. Furthermore, our results are consistent with the pharmacological relevance of piperlongumine and could explain its anti-tumor effects previously reported. Our findings indicate that selective inhibition of p65 nuclear import may be a novel therapeutic strategy for ABC-LDBCL treatment.

2. Materials and Methods

2.1. Cell culture, antibodies and reagents

The OCI-Ly10, U2932 and DB cell lines were maintained in IMEM medium supplemented with 10% fetal bovine serum. Piperlongumine was obtained from Cayman Chemical (Ann Arbor, Michigan, USA). Antibodies against p21, pro-caspase9 and pro-caspase3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Bcl-2, survivin, Bax, p65 and p-p65 were purchased from Cell Signaling Technology (Beverly, MA). Alexa 488-conjugated donkey anti-mouse antibody was obtained from Invitrogen Life Technology (Invitrogen, Carlsbad, CA).

2.2. Apoptosis assays

To assess apoptosis, aliquots of 1×10^6 cells were plated in 24-well plates and incubated with medium alone or with serial dilutions of piperlongumine up to 24 h. Cells were harvested and stained with PE-conjugated annexin-V and 7-AAD (eBioscience, San Diego, CA). As a standard, 1×10^6/mL of cells per treatment condition were fixed and stained with 5 μL Annexin V-PE and 5 μL 7-AAD. The mixed solution was incubated in the dark at room temperature for 15 min, 400 μl of 1 × dilution buffer was then added to each tube and cell apoptosis analysis was performed by FACScan (Becton Dickinson). Data were analyzed by CellQuest software (Becton Dickson).

2.3. Luciferase reporter assays

HEK-293 T cells were plated onto 96-well dishes and incubated at 37 °C. Twenty-four hours after plating, pNF-κB-Luc and pRL-TK plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). All luciferase activities were normalized for transfection efficiency by cotransfection with pRL-TK Renilla luciferase vector. After an additional 16 h of culturing, cells were pretreated with or without piperlongumine for 4 h before stimulation with or without 1 nM of TNF-α for 24 h. The cells were then harvested and reporter activity was assayed using the Dual Luciferase Assay System (Promega, Madison, WI).

2.4. Western blotting

Cytoplasmic, nuclear and whole cell extracts of untreated and treated cells were used in Western blot analysis. For the preparation of whole-cell lysates, cells were washed with ice cold phosphate-buffered saline (PBS). Lysis buffer were then added to the cells and further incubated on ice for 10 min. The nuclear and cytosolic protein extracts were extracted using NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) according to the manufacturer' instructions. The protein extracts (30–100 μg) were subjected to Western blot analysis.

2.5. Immunofluorescence microscopy

To observe the localization of p65, 1.5×10^4 Hela cells were seeded onto black optical-bottom 96-well glass plates and growth overnight. Cells were transfected with Flag-p65 plasmid. After an additional 24 h of culturing, cells were pretreated with or without 4 μM of piperlongumine for 3 h before stimulation with or without 20 ng/ml of TNF-α for 30 min. Following the indicated treatments, cells were stained and analyzed using a fluorescence microscope (Olympus, Japan).

2.6. Mass spectrometric analysis

Piperlongumine binding to p65 peptide was analyzed by MALDI-TOF-MS in a linear model. The peptide MRFRYKCEGR (amino acids sequence 32–41 of p65) and its derivative with Cys38 substituted by Ser were synthesized (GeneScript, China). Piperlongumine (20 μg) was incubated with one microgram of each peptide in buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 50% methanol) overnight at 37 °C. After incubation, an aliquot of 0.5 μL of this solution was mixed with 0.5 μL of the matrix solution. This sample was analyzed with a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, USA).

2.7. Data analysis

Data are means and standard deviations of three independent experiments with three to five replicates each. The results were statistical analyzed using a Student's t test and considered statistically significant at the $p < 0.05$ level.

3. Results

3.1. Piperlongumine selectively inhibits proliferation and induces apoptosis in ABC-DLBCL cell lines

To determine whether piperlongumine more selectively suppress ABC-DLBCL cells than GCB-DLBCL, ABC and GCB subtype cells were exposed to increasing concentrations of piperlongumine. Piperlongumine induced significant selective dose-dependent suppression of ABC-DLBCL cells (Fig. 1B). Strikingly, 2 μM of piperlongumine caused about 40% decrease of cell viability in ABC-DLBCL cells, but without significantly affecting GCB-DLBCL cells. Furthermore, the reduced viability was much more pronounced in ABC-DLBCL cells, while GCB-DLBCL cell viability was only slightly impaired at the high concentration. To evaluate whether the effects of piperlongumine on cell viability could be related to apoptosis,

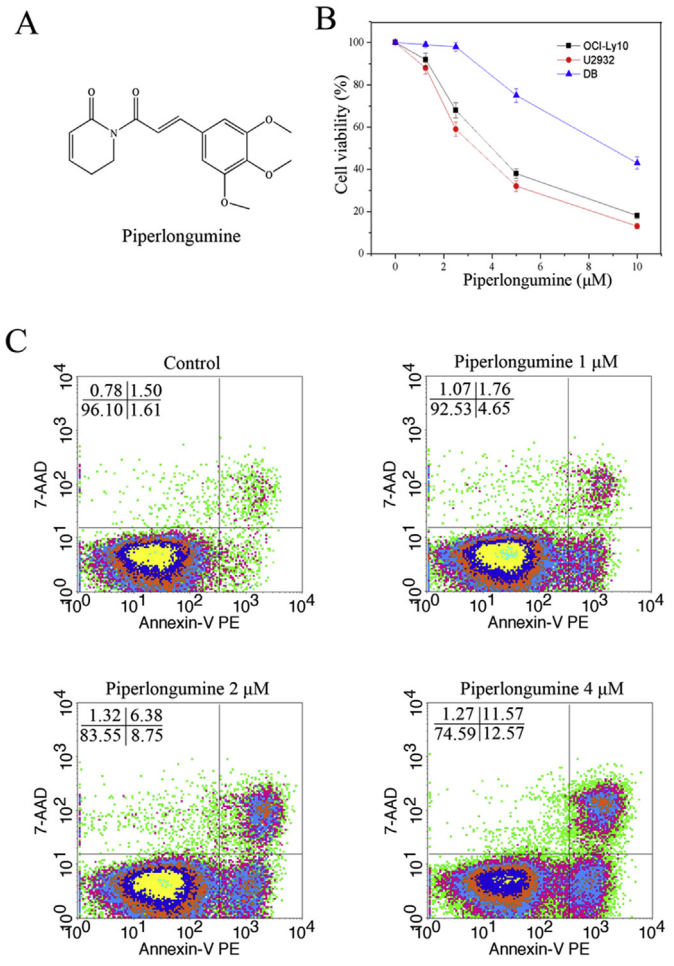

Fig. 1. Piperlongumine selectively induces cytotoxic effects in ABC-DLBCL cell lines. (A) Structure of piperlongumine. (B) Inhibition of the cell growth of ABC-DLBCL cells by piperlongumine. ABC-DLBCL cells were treated with the indicated concentrations of piperlongumine for 48 h. (C) Flow cytometric analysis of ABC-DLBCL cells treated with piperlongumine. ABC-DLBCL cells were treated with the indicated concentrations of piperlongumine for 24 h and cells were subsequently stained with PE-conjugated annexin-V and 7-AAD and analyzed by flow cytometry.

flow cytometric analysis was performed. As shown in Fig. 1C, piperlongumine treatment resulted in apoptosis in a dose dependent manner. The percentage of early and late apoptosis at 1, 2 and 4 μM piperlongumine ranged between 6 and 24% in OCI-Ly10 cells (Fig. 1C). Taken together, these results show that piperlongumine induces a selective cytotoxic effect in ABC-DLBCL cells.

3.2. Piperlongumine suppresses expression of NF-κB regulated survival and apoptotic proteins

Because NF-κB is involved in the regulation of cell survival proteins, we examined whether piperlongumine can modulate the expression of these survival and apoptotic proteins. We found that piperlongumine down-regulated the expression of cell survival proteins (Bcl-2 and surviving) and up-regulated the apoptotic proteins (Bax and p21) both in a dose-dependent manner in OCI-Ly10 and U2932 cells (Fig. 2A and B).

3.3. Piperlongumine inhibits TNF-α induced NF-κB activation and nuclear translocation of p65 in ABC-DLBCL cells

To demonstrate that decreased viability of ABC-DLBCL cells after piperlongumine treatment is linked to NF-κB inhibition, we first investigated whether piperlongumine can affect TNF-α induced NF-κB transcriptional activity. The inhibitory effect of piperlongumine on NF-κB activity was examined by performing a reporter assay with the use of 293 T cells transiently transfected with NF-κB-luc reporter plasmid (Fig. 2C). Luciferase activity, which represents transcriptional activation of NF-κB, was 18 fold greater induction by TNF-α than by a vector control. When we pretreated the cells with piperlongumine, the TNF-α induced NF-κB transcriptional activity was inhibited in a dose-dependent manner.

We then examined whether piperlongumine affects TNF-α induced nuclear translocation. Western blot analysis showed that TNF-α induced nuclear translocation of p65 in a time-dependent manner in OCI-Ly10 and U2932 cells (Fig. 2D and E). When the cells were pretreated with piperlongumine, TNF-α failed to induce nuclear translocation of p65. TNF-α induces the phosphorylation of p65, which is required for its transcriptional activity. TNF-α induced p65 phosphorylation in the nuclear in a time-dependent manner. In cells treated with piperlongumine, TNF-α failed to induce p65 phosphorylation. Similar results were obtained with cytoplasmic p65 phosphorylation.

3.4. Piperlongumine directly binds to cysteine 38 of NF-κB p65 subunit

It has been shown that the cysteine residue located at position 38 in p65 is highly susceptible to various agents [13]. To investigate whether piperlongumine could bind directly to Cys38 of p65, we performed a mass spectrometry analysis of a synthetic p65 peptide. The wild type peptide containing Cys and the mutant peptide containing Ser at the position corresponding to Cys38 of p65 were synthesized and reacted with piperlongumine. As shown in Fig. 3A, the p65 wild type peptide showed a major peak at m/z 1345.671, which corresponds to the calculated molecular mass of the p65 peptide. Upon incubation with piperlongumine, the major peak of p65 peptide was shifted to m/z 1662.847 (Fig. 3B). The mass shift between the modified peptide and the parental one was 317, which corresponds to the molecular weight of piperlongumine, indicating that piperlongumine can bind covalently to p65. However, no peak corresponding to a piperlongumine adduct could be observed for the sample derived from the mutant peptide treated with piperlongumine, suggesting that the active site Cys38 residue is the main target of modification by piperlongumine (Fig. 3D).

3.5. Mutation of Cys38 in p65 attenuates the inhibitory effect of piperlongumine

To further prove piperlongumine regulates NF-κB activation via directly target p65, we performed immunofluorescence experiments to analyze the subcellular localization of wild type and mutant p65 in cells treatment with piperlongumine. The cells expressing wild type and mutant p65 both showed a cytoplasmic localization, demonstrating that exogenous wild or mutant type p65 did not alter its subcellular localization and activity. Upon addition of TNF-α to the cells, both wild and mutant p65 localization were shifted towards the nucleus. However, piperlongumine could not inhibit the TNF-α induced nuclear translocation of p65C38S, suggesting that piperlongumine appears to target cysteine 38 of p65 (Fig. 4A).

To confirm the results observed by immunofluorescence analysis, we investigated whether the p65 mutant could reverse the inhibition effect of piperlongumine on p65 nuclear translocation using Western blot. The TNF-α induced nuclear translocation of wild type p65 was efficiently impaired in the presence of piperlongumine. However, cells expressing p65-C38S mutant were resistant to the treatment of piperlongumine, as mutant p65 was

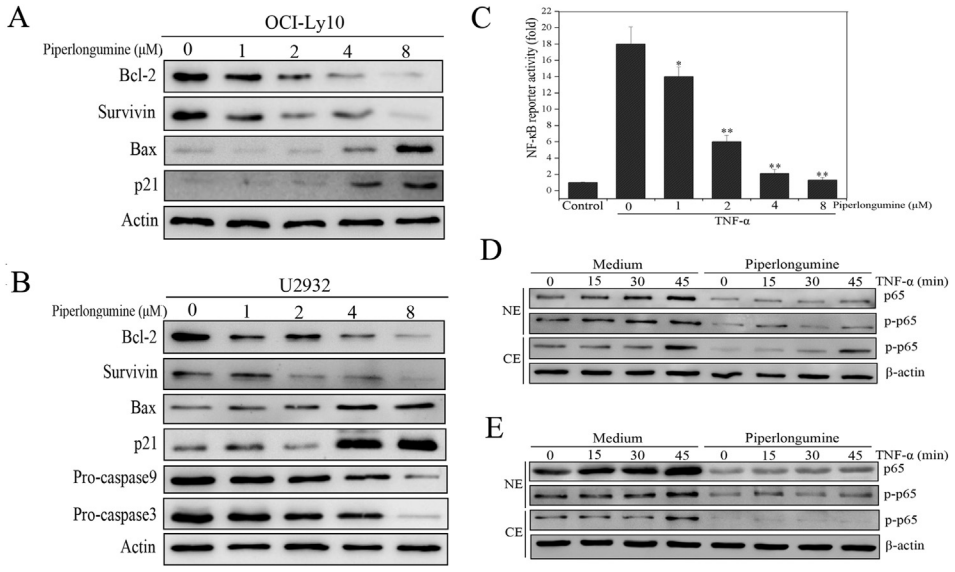

Fig. 2. Piperlongumine inhibits TNF-α induced nuclear translocation and regulates the expression of NF-κB dependent gene products. OCI-Ly10 cells (A) or U2932 cells (B) incubated with indicated concentrations of piperlongumine for 24 h. Whole cell extracts were prepared and subjected to Western blot analysis using the relevant antibodies. (C) Piperlongumine suppresses NF-κB reporter gene expression induced by TNF-α. 293 T cells were transfected with NF-κB-luc and pRL-TK plasmids. After transfection, the cells were pretreated with or without piperlongumine for 4 h before stimulation with or without 1 nM of TNF-α for an additional 24 h. Cell supernatants were assayed for NF-κB transcriptional activity. (D) Piperlongumine inhibits TNF-α induced nuclear translocation and phosphorylation of p65 in OCI-Ly10 cells. Cells were pretreated or untreated with 4 μM piperlongumine for 3 h and then treated with 0.2 nM TNF-α. The nuclear extracts (NE) and cytoplasmic extracts (CE) obtained from the cells were analyzed by Western blotting. (E) Piperlongumine inhibits TNF-α induced nuclear translocation and phosphorylation of p65 in U2932 cells same with above.

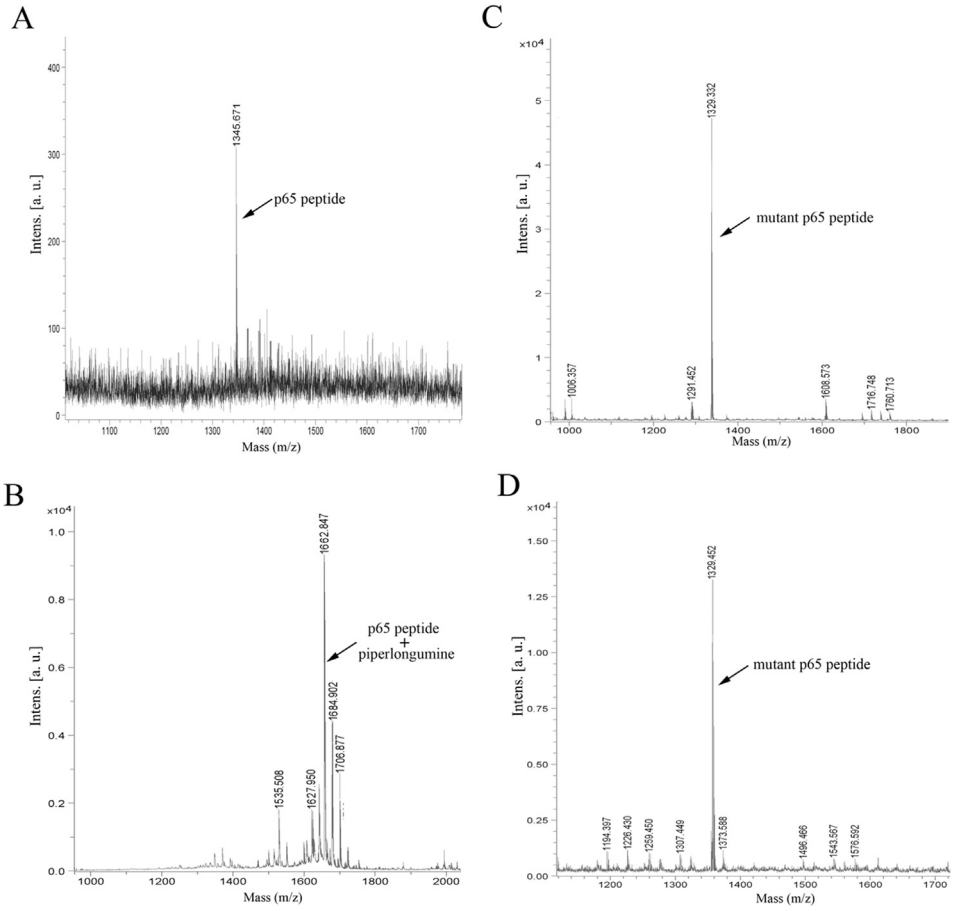

Fig. 3. Piperlongumine directly binds to Cys38 of a p65 peptide. Synthetic peptides containing Cys38 of p65 (A) or the mutant peptides in which Cys38 was substituted with Ser (C) were treated with piperlongumine (B, D) for 24 h and analyzed by MALDI-TOF MS.

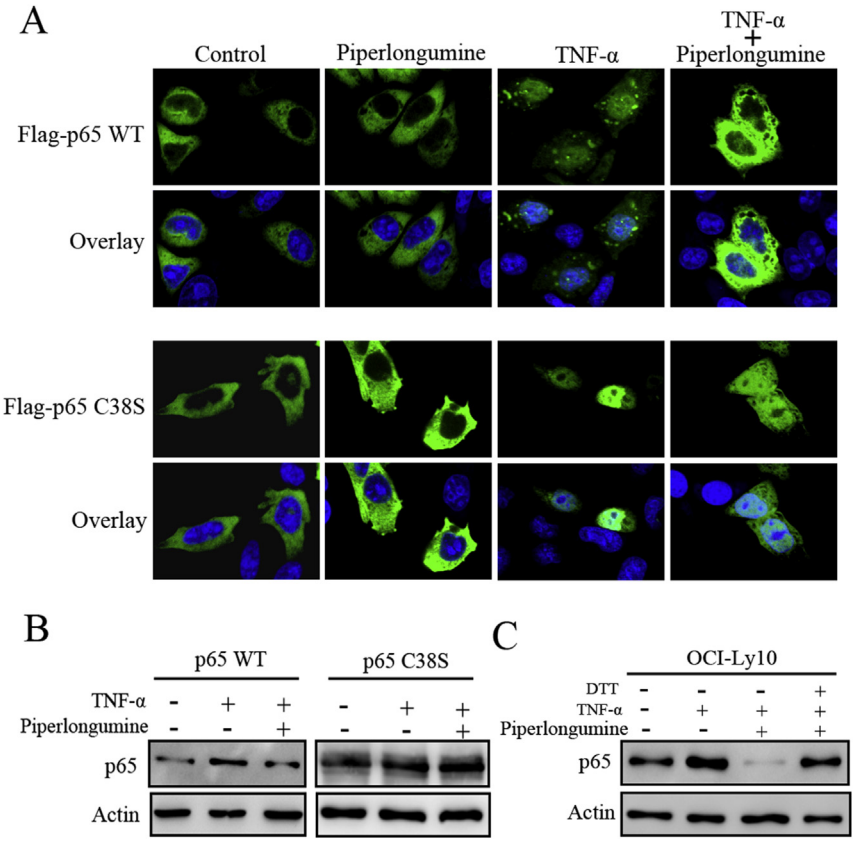

Fig. 4. Piperlongumine is ineffective in cells expressing mutant p65. (A) Fluorescence analysis the effect of piperlongumine on nuclear import of wild or mutant type of p65. Hela cells were transfected Flag-tagged wild type p65 or Cys38 mutant p65. After 24 h, cells were stimulated with or without 1 nM TNF-α for 30 min under pretreatment with or without 4 μM piperlongumine for 3 h. Then cells were fixed and stained with antibodies against the Flag tag. (B) Lack of effect of piperlongumine on nuclear import of mutant p65. 293 T cells were transfected Flag-tagged wild type p65 or Cys38 mutant p65. After 24 h, cells were stimulated with or without 1 nM TNF-α for 30 min under pretreatment with or without 4 μM piperlongumine for 3 h. The nuclear extracts obtained from the cells were analyzed by Western blotting. (C) DTT reverses the inhibitory effect of piperlongumine on p65 nuclear import. OCI-Ly10 cells were incubated with 4 μM piperlongumine with or without 100 μM DTT for 2 h and then stimulated with or without 0.2 nM TNF-α for 30 min. The nuclear extracts obtained from the cells were analyzed by Western blotting.

still showed a nuclear localization (Fig. 4B). To obtain further support for the above notion, we examined the effect of DTT, a potent reducing agent, which protects thiol groups in proteins like cysteine from oxidization, on the effect of piperlongumine. Pretreatment of cells with DTT, which may reduce reactive cysteine residues in the target proteins, inhibited the effect of piperlongumine (Fig. 4C).

4. Discussion

In this study, we demonstrated that piperlongumine could selectively kill ABC-DLBCL through directly inhibiting NF-κB p65 subunit nuclear import. This is the first report to suggest that piperlongumine could directly bind to p65 and specifically suppress NF-κB activation. We found that piperlongumine suppressed the NF-κB activation induced by TNF-α, and that the inhibition of NF-κB was due to binding to p65, leading to the suppression of p65 nuclear translocation and phosphorylation. Our current study provides new insight into piperlongumine's mechanism of action and novel approach to ABC-DLBCL target therapy.

NF-κB pathway has a well-established role in cancer biology. In particular, genetic and molecular alterations of NF-κB family members and their transcriptional target genes have been implicated in the development of ABC-DLBCL [14]. The central role of NF-κB in proliferation and survival make it an ideal candidate for therapeutic targeting in ABC-DLBCL. Unfortunately, the diverse role it plays in normal cellular functions provides substantial risk for widespread toxicity to patients [15]. However, in nuclear extracts of ABC-DLBCLs, NF-κB exhibited high levels of activity versus normal PBMCs, in which activity was rarely exhibited [16]. These reports indicate that selective inhibition of p65 nuclear import may be a promising strategy for the treatment of ABC-DLBC with minimizing toxic effects on normal cells. Our results demonstrated that piperlongumine could directly suppress p65 nuclear import and selectively inhibit the proliferation of ABC-DLBCL cells. More importantly, it has been reported that piperlongumine could selectively kill cancer cells and did not affect noncancerous cell types even at high doses. Piperlongumine can be administered orally and show good tolerability in animal models. Taken together, these studies demonstrate that selective inhibition of p65 nuclear import may have low toxic effects on normal cells.

Chronic activation of the BCR pathway in ABC-DLBCL is mediated by several different mechanisms, many of them upstream of NF-κB [17]. However, most oncogenic mutations in ABC-DLBCL occur between upstream kinases and NF-κB [18]. This raise a question that the upstream protein kinases may not be optimal targets. Thus, selectively suppression of the NF-κB activity would likely be necessary for maximal anti-lymphoma activity. Our results demonstrate that piperlongumine can both kill ABC-DLBCL cell line (OCI-Ly10) with CD79 A/B and MYD88 mutations, but also U2932 cells without these mutation. Furthermore, low concentration of piperlongumine caused a decrease of cell viability in the ABC-DLBCL cells, without significantly affecting GCB-DLBCL

cells. Thus, directly targeting NF-κB p65 subunit nuclear import may possess some advantages over the inhibition of upstream protein kinases. This selective action of piperlongumine appears to be important for its specificity to exert anti-cancer activity, because NF-κB p65 subunit plays a major role in cancer cell growth.

How piperlongumine inhibit nuclear import of p65 is not yet clear and thus was investigated in detail. It is known that cysteine 38 in the p65 subunit of NF-κB participates in DNA bingding by forming a hydrogen bond with the phosphate backbone of the κB-DNA motif [19]. Our results demonstrate that when this Cys38 was replaced by serine in p65, piperlongumine failed to bind with p65 peptide and inhibit the nuclear import of p65. Thus, our results suggest that piperlongumine must modify this cysteine residue leading to NF-κB suppression. This inhibition could be reversed with the reducing agent DTT, which suggests that targeting p65 involves the interaction of piperlongumine with sulfhydryl group within p65. All these results suggest that piperlongumine is interacting with the cysteine residue of p65 directly.

In summary, we performed critical experimental to demonstrate that piperlongumine could target p65 nuclear localization and thereby modulate NF-κB activity. In particular, we have provided strong evidence in support of the mechanism of action for piperlongumine which involves regulation NF-κB dependent gene expression. In addition, effectivity and selectivity of p65 nuclear import inhibition by piperlongumine can be optimized by medicinal chemistry programs to yield a more potent and possibly selective drug for the treatment of ABC-DLBCL. Taken together, the results reported here identify piperlongumine as a lead compound targeting NF-κB p65 subunit and demonstrate the p65 nuclear import inhibition is a promising strategy for the treatment of ABC-DLBC.

Conflict of interest

None.

Acknowledgments

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